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Use of Wide-Host-Range Bacteriophages to Reduce *Salmonella* on Poultry Products

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Abstract: Bacteriophages used to treat infections are typically amplified in a pathogenic host. However, this practice introduces the risk of administering any remaining bacteriophage-resistant pathogen during bacteriophage application if separation techniques are less than perfect. In this study, bacteriophage isolates capable of replicating in both *Salmonella* and *Klebsiella oxytoca* were identified and applied to poultry carcasses. These Wide-Host-Range bacteriophages (WHR) were amplified using the non-pathogenic bacteria, *Klebsiella oxytoca* in tryptic soy broth until a titer of ~10^9 PFU/mL was obtained. WHR and *Klebsiella oxytoca* were not separated prior to treatment of carcasses. Fresh processed chicken carcasses were inoculated with either *Salmonella enteritidis* (SE) or *S. typhimurium* (ST), sprayed with 5 mL of WHR and rinsed with sterile water. Samples were enriched, plated on XLD agar and evaluated for *Salmonella*-typical colonies. In four separate trials, WHR significantly reduced the recovery of SE. No SE was detected in two trials and a greater than 70% reduction was seen in the other two trials. ST was also significantly reduced in the two trials in which it was included ($p<0.05$). These experiments suggest that WHR could be an inexpensive and safe method for the reduction of *Salmonella* on broiler carcasses.

Key words: Bacteriophages, *Salmonella*, poultry products, host range

Introduction

Many of the 1.4 million annual cases of paratyphoid *Salmonella* in the United States are associated with contaminated poultry and eggs (FoodNet, 2002), for this reason, controlling paratyphoid *Salmonella* infections has become an important objective for the poultry industry. Since 1996, the National Antimicrobial Resistance Monitoring System for Enteric Bacteria has identified increasing numbers of *Salmonellae* isolates that are resistant to multiple antimicrobial agents (NARMS, 2001). Recently, the United States Food and Drug Administration (FDA) proposed the withdrawal of flouroquinolones in poultry production because of the increase in resistant *Campylobacter* infections in humans (Schwetz, 2002). This leads to fewer options for poultry companies to use when treating *Salmonella*-infected poultry flocks prior to processing. Thus, development of alternatives to antibiotics is becoming increasingly important.

Much recent research utilizing bacteriophages has focused on treating enteric and respiratory infections in livestock and poultry (Smith and Huggins, 1982, 1983; Slopek *et al*., 1985; Smith *et al*., 1987; Huff *et al*., 2005). However, some recent publications have demonstrated that bacteriophages can be used to successfully treat food products (Atterbury *et al*., 2003; Leverentz *et al*., 2001; O'Flynn *et al*., 2004; Higgins *et al*., 2005). In 2003, Atterbury and coworkers reduced levels of *Campylobacter* on chicken skin, while Leverentz *et al.* (2003) reported a reduction in *Listeria* on fresh-cut produce. O'Flynn *et al*. (2004) selected bacteriophages that successfully reduced the incidence of *E. coli* O157:H7 on beef. Higgins *et al*. (2005) recently reported the ability to select for bacteriophages that could reduce the incidence of *Salmonella* recovery on processed broiler and turkey carcasses.

In each of the above-mentioned studies, bacteriophages were amplified in the pathogenic host. While these bacteriophages worked well, they would be difficult to use in processing plants where the strain of bacteria could be different from flock to flock. An important consideration in the use of bacteriophages for reduction of pathogens is the necessity to ensure an absence of harmful bacteria in the bacteriophage preparation. It is common in the amplification of bacteriophages to have bacterial cells remaining that have become resistant to lysis by those bacteriophages. In order to ensure that all bacterial cells are removed from the bacteriophage preparation, costly filtration, centrifugation and/or chemical treatment steps must be employed and even with screening methods it could be difficult to identify small numbers of remaining bacteria in large volumes of bacteriophage filtrate. Here, we investigated the use of a non-pathogenic host that could be safely used to amplify bacteriophages. A non-pathogenic host would not necessarily require 100% effective filtration techniques to ensure safety of the bacteriophages, increasing the safety of the bacteriophage preparations while potentially reducing the cost of bacteriophages for application to food products.

Materials and Methods

Bacteriophage isolation: The isolation of wild-type bacteriophages and subsequent selection for WHR bacteriophages has been described (Higgins *et al*., 2005). Briefly, wastewater samples were obtained from a local municipal wastewater treatment plant and filtered through a 0.2 µm filter[1]. A combination of 100 µL of 10^7 cfu/mL *Salmonella enteritidis* PT 13A (SE) and 1 mL of the wastewater sample filtrate was added to 1.5 mL of Tryptic Soy Agar[2] (TSA) and poured over a warm TSA petri plate. Plates were incubated overnight at 37°C and those with confluent lysis of SE were then flooded with 15 mL of sterile 0.9% NaCl (saline). The fluid was poured off the plate and filtered through a 0.2 µm filter. Serial ten-fold dilutions were made in saline. Plates were poured as described above with 1 mL of each bacteriophage dilution. Individual distinct plaques resulting from this plating were then differentiated on the basis of plaque morphology and different plaques were sequentially passed on TSA plates at least three subsequent times to establish bacteriophage isolate purity.

Wide-Host-Range bacteriophages (WHR) were determined by amplifying bacteriophages in apathogenic bacteria of enteric origin (Bielke *et al*., 2003), including the genera *Escherichia*, *Citrobacter*, *Klebsiella* and *Kluyvera*. A single bacteriophage able to amplify in *Klebsiella oxytoca* (KO), isolated in our laboratory from the gastrointestinal tract of a healthy chicken, was selected for further testing. This bacteriophage isolate was also determined previously to lyse 8 different species of *Salmonella* (Hargis *et al*., 2007).

***Salmonella* amplification:** A primary poultry isolate of *Salmonella enteritidis* PT13A (SE) was obtained from the USDA National Veterinary Services Laboratory. This isolate was resistant to novobiocin[3] (NO; 25 µg/mL) and was selected for resistance to naladixic acid[4] (NA; 20 µg/mL) in our laboratory. *Salmonella typhimurium* (ST) was also obtained from the USDA NVSL and is similarly resistant to NO and NA. For these studies, both SE and ST were grown overnight is Tryptic Soy Broth[5] (TSB) at 37°C. Cells were washed 3 times in sterile saline by centrifugation at 1,864xg and the concentration was estimated with a spectrophotometer using a previously generated standard curve, to approximately 10^8 cfu/mL in sterile saline and then diluted to inoculated concentrations as described below. Concentrations of SE and ST were retrospectively determined by spread plating on XLD agar containing NO (25 µg/mL) and NA (20 µg/mL), followed by enumeration for each experiment. Actual determined colony-forming units for each experiment are reported.

Bacteriophage amplification: Bacteriophages used in these experiments were amplified in broth using a ratio of 1:3:5 (bacteriophage:KO:TSB) and were incubated at 37°C for 1.5 h. The bacteriophage culture was filtered through a 0.2 µm syringe filter and added to a fresh culture of KO, used in some laboratories as a precursor to sauerkraut fermentation (Lindquist, 2005) and TSB for incubation at 1.5 h. This process was repeated until the tube of bacteriophages and KO remained clear after incubation, which is an indication of high bacteriophage titer. Bacteriophages were quantitated for each experiment as described above.

Inoculation, treatment and culture of broiler carcasses: For all experiments, commercially processed broiler carcasses were removed from the processing line immediately prior to chill tank immersion. Each carcass was placed in an individual carcass rinse bag[6] (35×48 cm), sealed with a cable tie and randomly assigned to treatment groups. Carcasses were transported to the laboratory on ice and each carcass was inoculated with SE (all experiments) or ST (experiments 3 and 4) according to the methods described by Cox *et al*. (1981). Briefly, a sterile cell spreader, such as used for spread-plate enumeration, was used to thoroughly spread 100 µL of inoculum over the entire breast area. Each broiler carcass was then placed back into its original bag and stored at 4°C for 2 h. Carcasses were then individually removed from each bag and sprayed with 5.5 mL of sterile saline alone (controls) or saline containing the WHR, amplified as described above. Hand-held garden sprayers were calibrated to apply a fine mist of treatment over the breast area (5.5 mL/carcass). Numbers of SE (40 to 110 cfu) and ~10^9 PFU/carcass of bacteriophages for each experiment. One hundred mL of sterile water was then added to the abdominothoracic cavity of each carcass, the bag was sealed with a cable tie and the contents were vigorously shaken by hand for 30s. The rinse fluid was aseptically collected in sterile sealed containers[7] and an equal volume of concentrated (2x) tetrathionate broth base was added to the rinse fluid of each sample and samples were enriched and plated for *Salmonella* recovery as described above. Ten carcasses were used as negative controls to ensure that inoculated carcasses did not contain a NO/NA-resistant field isolate of *Salmonella*.

Enrichment and SE and ST Recovery: For all experiments, 50 mL of concentrated (2X) tetrathionate broth[8] was added to each rinse water sample and incubated overnight at 37°C. Samples were streaked for isolation on XLD agar plates containing NO (25 µg/mL) and NA (20 µg/mL) and were incubated overnight at 37°C. Each plate was evaluated for the presence or absence of lactose negative colonies with morphology consistent with the antibiotic-resistant *Salmonella*.

Table 1: Recovery (positive/total) of *Salmonella enteritidis* (SE) or *Salmonella typhimurium* (ST) from broiler carcasses treated with spray application of 5.5 mL of wide-host-range bacteriophage (WHRN)

	SE		ST	
	Control[1]	Treated	Control	Treated
Exp. 1[2]	18/20	5/20*	ND[3]	
Exp. 2[4]	20/20	2/20*	ND	
Exp. 3[5]	19/20	0/20*	18/20	2/20*
Exp. 4[6]	15/20	0/20*	15/20	8/20*

[1]all negative controls tested were negative for *Salmonella* recovery; [2]40 cfu SE, 2.9 x 10^9 PFU WHRN/carcass; [3]Not Done; [4]110 cfu SE, 6 x 10^9 PFU WHRN/carcass; [5]41 cfu SE, 31 cfu ST, 2.1 x 10^9 PFU WHRN/carcass; [6]51 cfu SE, 3 cfu ST, 1.7 x 10^9 PFU WHRN/carcass; *values are significantly different ($p<0.05$) within bacterial inoculum and within experiments

Statistical analysis: *Salmonella* recovery within inoculum species and within experiments were compared using the chi-squared test of independence (Zar, 1984) to determine significant ($p<0.05$) differences.

Results and Discussion

Table 1 summarizes the results of the recovery (positive/total) of SE or ST from broiler carcasses treated with spray application of 5.5 mL of WHR. Application of bacteriophages amplified in KO significantly reduced recovery of SE from inoculated carcasses in 4 consecutive replicates. Likewise, ST was significantly reduced by bacteriophage application in experiments 3 and 4. In experiment 1, recovery of SE was significantly reduced from 18/20 SE positive samples in the control group to 5/20 positive samples in the treated group, a difference of 65%. Experiment 2 also resulted in a significant reduction of SE, with a 90% reduction of SE recovered on treated vs. control carcasses. In Experiments 3 and 4, both SE and ST were reduced significantly. No SE was detected on bacteriophage-treated carcasses for both experiments. In Experiment 3, an 80% reduction of ST was observed and, a 35% reduction in recovery was noted for Experiment 4. The data from four experiments indicate that bacteriophages can successfully reduce the levels of SE and ST on processed broiler carcasses. Previous research (Higgins *et al*., 2005) indicated that only large numbers of bacteriophages could successfully reduce the levels of *Salmonella* on processed poultry. Therefore, only high levels (~10^9 PFU) of WHR were tested in these studies. The studies of Higgins *et al*. (2005) suggested that using 10^8 PFU of bacteriophages resulted in significantly less effectiveness than higher titers and arguments, to date, against using high levels of bacteriophages for carcass treatment are weak.

We did not investigate whether or not the *Salmonella* were immediately killed by bacteriophage treatment in these experiments. However, Goode *et al*. (2003) conducted similar experiments on broiler skin and found that the bacteriophages were, in fact, killing the *Salmonella* on the skin rather than during enrichment. Also, bacteriophages have been shown to effectively lyse other host bacteria over time in cold temperatures (Kudva *et al*., 1999), suggesting that the levels of *Salmonella* could be reduced by bacteriophages during proper temperature storage. As mentioned above, other investigators have successfully treated meat with lytic bacteriophages (Goode *et al*., 2003; O'Flynn *et al*., 2004; Higgins *et al*., 2005). Each group of scientists were able to significantly reduce pathogen levels, suggesting bacteriophages may be an attractive alternative to some chemical intervention strategies. WHR bacteriophages could be easier to use in commercial applications than the traditional host-specific bacteriophages (Jensen *et al*., 1998). This is largely due to the fact that a host-specific bacteriophage, treatment would require identification of the pathogen, selection of a suitable bacteriophage, safety testing of the bacteriophage and finally, amplification of the bacteriophage prior to application. WHR would allow for the development of a small library or cocktail of bacteriophages capable of killing common *Salmonella* isolates found in processing plants and applied without prior knowledge of which strain had infected the live poultry. With this scenario, safety testing and amplification would be completed long before birds were identified as *Salmonella* positive. WHR may also offer added safety to food producers, if amplified in apathogenic host bacteria, due to the absolute assurance that no pathogenic bacteria could potentially contaminate the bacteriophage treatment. In these experiments WHR was amplified using *Klebsiella oxytoca*, a bacteria used in some laboratories as a precursor to sauerkraut fermentation (Lindquist, 2005). This relatively safe bacteria was able to amplify a bacteriophage that was originally isolated using SE and the bacteriophages were not separated from the *Klebsiella* prior to application to the processed carcasses.

Application of bacteriophages in the processing plant to the surface of product rather than pre-slaughter to live animals provides potential benefits. First, bacteria entering the processing facility will be mostly naïve to the bacteriophages. Secondly, any potentially bacteriophage resistant organisms remaining on a product are unlikely to be re-introduced into poultry farms, retaining the naivete of bacteria entering the plant. Finally, treatment of enteric disease in live animals using bacteriophages is complicated due to the dynamic nature of the gastrointestinal tract and application of bacteriophages to the product is a very direct way to eliminate or greatly reduce food-borne pathogens. The results of the present study suggest that WHR could be an inexpensive and safe method for the reduction of *Salmonella* on broiler carcasses.

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